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Validity of putative calcium binding loops of photoprotein aequorin

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Three peptides containing the putative Ca^{2+} binding loops, I, II and III, respectively, of a photoprotein, aequorin, from jellyfish *Aequorea victoria* were synthesized by a solid-phase procedure. The peptides bound Ca^{2+} with dissociation constants of 10^{-3} to 10^{-4} M, providing evidence for the assumption that Ca^{2+} binding loops are actually responsible for the binding of Ca^{2+}. When the highly conserved 6th glycine residue in the 12-residue loops was replaced by arginine, no large effect was observed on Ca^{2+} binding. Exposure to a hydrophobic environment and the binding of Ca^{2+} brought about conformational changes to the peptides.

Aequorin; Calcium binding loop; Solid-phase synthesis; Dissociation constant; Conformation; *Aequorea victoria*

1. INTRODUCTION

A photoprotein, aequorin, of jellyfish *Aequorea victoria* occurs as a complex consisting of apoaequorin, coelenterazine and molecular O_2, and Ca^{2+} triggers light emission [1]. Apoaequorin has been assumed to have three Ca^{2+} binding loops, I, II and III, on the basis of their high sequence homologies to the Ca^{2+} binding loops I, III and IV, respectively, of bovine calmodulin [2] (Fig. 1). In the Ca^{2+} binding proteins, such as calmodulin [3], troponin C [4] and parvalbumin [5], the Ca^{2+} binding loops consist of 12 amino acid residues and are flanked by two helices at both ends (EF-hand structure). The highly conserved 6th glycine residue in the loops has appeared to be crucial for constructing a conformation suitable for chelating Ca^{2+} [6,7]. In the present study, a synthetic approach was undertaken in order to investigate whether the putative Ca^{2+} binding loops in apoaequorin actually interact with Ca^{2+} and whether the 6th glycine residue in the loops is essential for the binding of Ca^{2+}. For this purpose, peptides 1 (positions 20–40), 2 (positions 113–135) and 3 (positions 149–170) that cover the loops I, II and III, respectively, were synthesized. Peptides 1′ and 2′, which contain arginine in place of the 6th glycine residue in the loops, were also prepared.

Abbreviations: Boc, *t*-butoxycarbonyl; Pam, phenylacetamidomethyl; HF, anhydrous hydrofluoric acid; CD, circular dichroism; DMSO, dimethyl sulfoxide; TFE, trifluoroethanol; Tns, 2-toluidinylnaphthalene-6-sulfonate.

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2. EXPERIMENTAL

2.1. *Protected peptidyl-4-(oxymethyl)phenylacetamidomethyl resins*

Boc-amino acid-4-(oxymethyl)phenylacetamidomethyl (Pam) resins (0.43 mmol Ala/g and 0.39 mmol Leu/g) were prepared from aminomethylated copoly(styrene-divinylbenzene) (1%) resin (200–400 meshes) and Boc-amino acid-4-(oxymethyl)phenylacetic acid according to Mitchell et al. [8], followed by acetylation of the remaining amino groups with acetylimidazole. Successive additions of Boc-amino acids to Boc-Ala- or Boc-Leu-4-(oxymethyl)-Pam resin (2.0 g) along the sequences were conducted on a Beckman 990E peptide synthesizer according to the method of Wong and Merrifield [9] with the use of benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate and 1-hydroxybenzotriazole as the coupling reagents [10]. The side chain-protected amino acids employed were *β*-benzylaspartate, methionine sulfoxide, N^{g}-tosylarginine, N^{im}-benzyloxymethyl-histidine, N^{ε}-2-chlorobenzyloxycarbonyllysine, *O*-benzylserine, *O*-benzylthreonine, *O*-benzyltyrosine, N^{in}-formyltryptophan and *S*-acetamidomethylcysteine.

2.2. *Peptide hydrochlorides*

Protected peptidyl-4-(oxymethyl)-Pam resins (500 mg) were cleaved with HF (8.5 ml) in the presence of *p*-cresol (1 ml) and dimethyl sulfide (0.5 ml) for peptides 1 and 1′ or of anisole (1 ml) and 1,2-ethanedithiol (0.5 ml) for peptides 2, 2′ and 3 at 0°C for 3 h. No cleavage of the *S*-acetamidomethyl group in peptide 3 was made. After removal of HF and extraction with ethyl acetate, crude peptide was extracted with aqueous acetic acid containing HCl and purified by chromatography on a Sephadex G-25 column with aqueous acetic acid, followed by HPLC on a TSKgel ODS-120T column using a solvent system of 0.1% trifluoroacetic acid–acetonitrile containing 20% of 0.1% trifluoroacetic acid. The amino acid compositions of the hydrolysates of purified peptides were well in accord with the theoretical values.

2.3. *Determination of dissociation constants*

The dissociation constants for the binding of Ca^{2+} to peptides have been determined potentiometrically with an Orion 93-20 calcium ion-selective electrode connected to an Orion 701A ion meter at pH 8.0 (0.05 M Tris-HCl containing 0.1 M NaCl) and 25°C. The Nernstian slope of the electrode was in good agreement with the theoretical value. The solution of peptides were titrated with $CaCl_2$ and, at each

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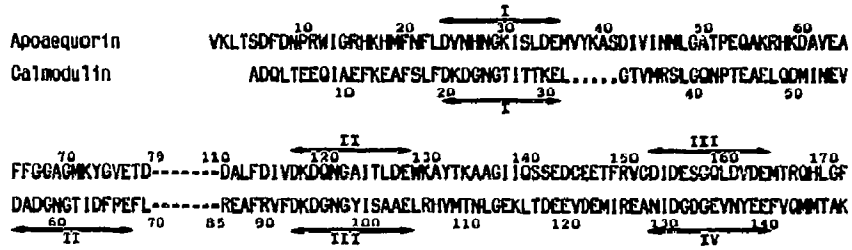

Fig. 1. Aligned amino acid sequences of apoaequorin and bovine calmodulin. The intermediate portions have been removed and dots in the calmodulin sequence represent absence of amino acid residues when compared with the corresponding segment of apoaequorin. The Ca^{2+} binding loops of calmodulin and the assumed Ca^{2+} binding loops of apoaequorin are indicated by arrows and numbered.

equilibrium state, the free Ca^{2+} concentration was calculated by use of the calibration graphs obtained immediately before and after titration.

2.4. *Circular dichroism of peptides*

Circular dichroism (CD) spectra for peptide 1 and 1′ were taken on a JASCO J-720 spectropolarimeter using a cell of 0.1 cm path-length at 20°C. Peptide solution (0.1 ml) in dimethyl sulfoxide (DMSO) was mixed with 1.0 ml of 10 mM Tris-HCl (pH 8.0), with or without 11 mM $CaCl_2$, or mixed with 1.0 ml of the mixture of 10 mM Tris-HCl buffer (pH 8.0), with or without 37 mM $CaCl_2$ and trifluoroethanol (TFE) (3:7). The helix contents were calculated according to the method of Chen et al. [11].

2.5. *Titration of 2-toluidinylnaphthalene-6-sulfonate with peptides*

2-Toluidinylnaphthalene-6-sulfonate (Tns) (15 μM) was titrated with increasing concentrations of peptide at pH 8.0 (50 mM Tris-HCl containing 0.1 M NaCl) and 25°C, and fluorescence spectra were recorded on a JASCO FP-550A spectrofluorometer with excitation at 365 nm for Tns. Tns–peptide complexes were titrated with increasing concentrations of Ca^{2+} under the same conditions as above.

3. RESULTS AND DISCUSSION

The dissociation constants (K_d) of peptides for Ca^{2+} were estimated by determining the free Ca^{2+} concentration with a calcium ion electrode. The fractions of peptide in the Ca^{2+}-chelated state were measured against increasing concentration of Ca^{2+}. The sigmoidal curves were obtained and the bound Ca^{2+} concentration never exceeded the peptide concentration (Fig. 2), indicating that the interaction of peptide and Ca^{2+} is a one-to-one stoichiometry. The following equation holds:

$$f = [Ca^{2+}]/(K_d = [Ca^{2+}])$$

where f is the fraction of peptide in the Ca^{2+}-chelated state and $[Ca^{2+}]$ the free Ca^{2+} concentration. Thus, K_d values were evaluated as $\log K_d = \log[Ca^{2+}]$ where the average number of bound Ca^{2+} per one peptide molecule was 0.5 (Table I). Peptides 1, 2 and 3 can bind Ca^{2+} with magnitudes of 10^{-3} to 10^{-4} M. These K_d values are approximately 10^3–10^4-fold greater than that (1.4×10^{-7} M, pH 7.0), reported for apoaequorin [12]. However, it is noted that the K_d values were comparable to that (7.4×10^{-4} M) reported for a synthetic 33-residue peptide of bovine brain calmodulin Ca^{2+} binding site III [13]. Since the present study mainly focused on the loop portions of the helix-loop-helix EF-hand structures, the peptides prepared contained only N-terminal 4 and C-terminal 5–7 residues at both sides of the central 12-residue peptides. Abilities to form helix, if any, at both ends may be to small extents only. Taking such circumstances into consideration, the data obtained would provide strong evidence for putative Ca^{2+} binding sites of apoaequorin actually being responsible for the binding of Ca^{2+}.

Analogs 1′ and 2′ that have arginine instead of the 6th glycine residue in the loops of peptides 1 and 2 were found to bind Ca^{2+} as effectively as the original peptides (Table I), indicating that such a substitution brings about no large effect on the Ca^{2+} binding. Although the

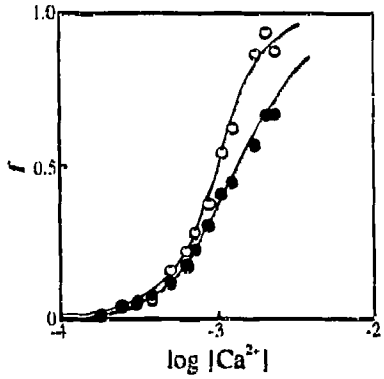

Fig. 2. Titration curves of peptides 1 (○) and 1′ (●) with Ca^{2+}. Peptide 1 (2.26×10^{-4} M) and peptide 1′ (1.98×10^{-4} M) were titrated with increasing concentrations of $CaCl_2$ at pH 8.0 (0.05 M Tris-HCl containing 0.1 M NaCl) and 25°C. The free Ca^{2+} concentrations were determined potentiometrically with a Ca^{2+} ion-selective electrode.

Table I

Dissociation constants of peptides for Ca^{2+} at pH 8.0

Peptide	Dissociation constant (M)
1	1.05×10^{-3}
1′	1.35×10^{-3}
2	0.63×10^{-3}
2′	0.98×10^{-3}
3	1.23×10^{-3}

peptides and proteins remains to be clarified.

CD spectra indicated that peptides 1 and 1′ are in random conformation in buffer both in the absence and presence of 10 mM Ca^{2+} (Fig. 3). However, in the presence of 70% TFE, conformations containing 8 and 20% α-helix were observed for peptides 1 and 1′, respectively (Fig. 3). The peptides appear to have the potential to form an α-helix, although partially, possibly at both

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